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OVER-EXPRESSION OF OXYTOCIN RECEPTORS IN THE HYPOTHALAMIC PVN INCREASES BRS AND BUFFERS BP VARIABILITY IN CONSCIOUS RATS

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Short title: PVN OT receptors in Autonomic Circulatory Control

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Summary

Background and purpose. The paraventricular nucleus (PVN) of the hypothalamus is an important integrative site of neuroendocrine control of the circulation. We investigated the role of oxytocin receptors (OTRs) in PVN in cardiovascular homeostasis. **Experimental approach**. Experiments were performed in conscious male Wistar rats equipped with radiotelemetric device. The PVN was unilaterally co-transfected with an adenoviral vector (Ad) engineered to over-express OTRs along with an enhanced green fluorescent protein (eGFP) tag. Control groups were PVN transfected with an Ad expressing eGFP alone or untransfected, sham injected wild-type rats (Wt). Rats were recorded without and with selective blockade of OTRs (OTX), both under baseline and stressful conditions. Baro-receptor reflex sensitivity (BRS) and cardiovascular short-term variability were evaluated using the sequence method and spectral methodology, respectively. **Key results**. Under baseline conditions OTR rats exhibited enhanced BRS and reduced BP variability in comparison to eGFP and Wt rats. Exposure to stress increased BP, BP variability and HR in all rats. In eGFP and Wt rats, but not in OTR rats, BRS decreased during exposure to stress. Pre-treatment of OTR rats with OTX reduced BRS and enhanced BP and HR variability under basliene and stressful conditions. In Wt rats pre-treated with OTX, BRS was suppressed and BP variability was increased under baseline and stress while HR variability was increased only during stress. **Conclusions and Implications**. OTRs in PVN are involved in tonic neural control of BRS and cardiovascular short-term variability. The failure of this mechanism could critically contribute to autonomic deregulation in cardiovascular disease.

Keywords.

Oxytocin receptor, paraventricular nucleus, adenoviral vector, baro-receptor reflex, blood pressure variability

Abbreviations.

BRR	baro-receptor reflex;
BRS	baro-receptor reflex sensitivity;
VLF	very low frequency short-term variability;
LF	low frequency short-term variability;
HF	high frequency short-term variability;
OTRs	Oxytocin receptors
OTX	Selective non-peptide oxytocin receptor antagonist;
PVN	paraventricular nucleus;
NTS	nucleus of the solitary tract;
NA	nucleus ambiguus;
DVN	dorsal nucleus of vagus;
RVLM	rostroventrolateral medulla;
IML	intermediolateral column of the spinal cord.

Introduction

In addition to its well established roles in reproduction and maternity, convincing evidence has accumulated in the last decades to suggest that oxytocin (OT), a peptide hormone mainly synthesized in the hypothalamic paraventricular (PVN) and supraoptic nuclei, is also involved in the control of the circulation. Peripherally, an independent OT system has been discovered in the heart and the blood vessels, and associated with heart development, heart renewal and natriuresis (Gutkowska and Jankowski, 2012; Japundzic-Zigon, 2013). OT has been shown to exert direct negative inotropic and chronotripic action on the heart (Costa-e-Sousa *et al*., 2005), to produce weak vasoconstriction (Suzuki *et al*., 1992) and NO dependent vasodilatation (Katusic *et al*., 1986).

In addition to its peripheral action, OT exerts endocrine and neuromodulator influences on the circulation (Haanwinckel *et al*.,1995; Randolph *et al*., 1998). OT neurons located in the parvocellular part of the PVN project to the brainstem vagal nuclear complex (nucleus tractus solitarius - NTS, nucleus ambiguus - NA and dorsal vagal nucleus – DVN), rostroventrolateral medulla (RVLM) and the intermediolateral column of the spinal cord (IML) where OT **influences** parasympathetic and sympathetic outflow to the heart and the blood vessels (Sawchenko and Swanson, 1982; Lang *et al*., 1983; Zerihun and Harris, 1983; Hosoya *et al*., 1995; Jansen *et al*., 1995; Hallbeck *et al*., 2001; Geerling *et al*., 2010). *In vivo* animal studies indicate that OT mediates the HR response to exercise (Martins *et al*., 2005) and HR adjustment to stress (Wsol *et al*., 2008). Reduction in OT mRNA in PVN and OT receptors mRNA in the brainstem was described in genetically hypertensive rats (Martins *et al*., 2005) while failure of central OT was associated with increased cardiovascular reactivity to stress in rats survivors of myocardial infarction (Wsol *et al*., 2009).

OT produces its effects by the stimulation of a specific OT receptor (OTR) well defined in terms of genes, protein structure and pharmacology (Rozen *et al*., 1995, Manning

et al., 2012). OTRs belong to G-protein receptor family coupled to phospholipase C signaling pathways, and are widely distributed at the periphery and the CNS (Freund-Mercier, *et al*., 1987; Gimpl and Fahrenholz, 2001). The focus of present work is the role of OTRs found in PVN which have been reported to play an important role in autoregulation of magnocellular neuronal activity (Richard *et al*., 1997). **We hypothesized that, by increasing the number of OTRs located in PVN and by selectively blocking their activity, we can modulate PVN neuronal activity involved in autonomic cardiovascular control. To test this hypothesis we used genetic tools, microinjection of adenoviral vectors (Ads) carrying the tagged gene for OTR into the PVN to induce over-expression of OTR and pharmacological tools, microinjcetions of selective OTR antagonist in PVN of conscious transfecetd and non-transfected wild type rats, both under baseline conditions and stress.**

Methods

All experimental procedures in this study conformed to European Communities Council Directive of November 24, 1986 (86/609/EEC). The experimental protocol was approved by the Faculty of Medicine University of Belgrade Ethics review board, and the results of the study are reported in accordance with The ARRIVE Guidelines.

Animals

Experiments were performed in male, twelve week old male Wistar rats weighing 310-360g bred at the local animal facility. Rats were housed individually in a controlled environment: 12h/12h light dark-cycle, **temperature 21 ± 2 °C and humidity 60 ± 5 %** with access to standard pelleted chows (0.2 % w/v sodium content, Veterinarski zavod, Subotica) and tap water *ad libitum*. The number of rats in each protocol was calculated statistically taking into account intra-group variability, using the 'Power Sample Size Calculation'

software available at: http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize for power of 90% and type I error probability of 0.05. At the end of the experiment, the rats were euthanized using a combination of three anesthetics (0.1 ml, i.p. of T61® solution).

Surgery

Rats underwent two surgical procedures at ten days interval. Under combined ketamine (100 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) anesthesia, a 3 cm-long medial abdominal incision was made and the intestine retracted to expose the abdominal aorta. The tip of the catheter of the radio telemetric probe (TA11-PA C40, DSI, St. Paul, MN, USA) was inserted into the aorta using a 21G needle. The inserted catheter was fixed with 3M Vetbond™ and tissue cellulose patch (DSI, St. Paul, MN, USA). The transmitter was attached to the anterior abdominal wall and the wound was closed by suture. To prevent bacterial infection neomycin and bacitracin were sprayed topically, and the rats were treated with gentamicin (25 mg/kg i.m.) three days before, and again on the day of surgery. To reduce pain, rats received carprofen (5 mg/kg/day, s.c.) on the day of surgery and for the next two days. Each rat was housed in a Plexiglas cage (30 cm x 30 cm x 30 cm) and left to **recover fully for 10 days.**

The second surgery was performed in wild type rats under the same anesthetics and postoperative care. Rats head was mounted in stereotaxic frame and the skin was incised 3 mm to expose the scull. A hole was opened with dental drill to position 23G guide above PVN (AP = 1.8 mm caudal from bregma, LAT = 0.4 mm from midline, 6.5 mm beneath the scull; Paxinos and Watson, 2005) and fixed with dental cement. On the day of experimentation 7.5 mm-long 30G needle was used for microinfusion of drugs into the PVN. At the end of experiment, rats' brain was removed. After the center of the microinfusion site was identified in the hypothalamus, the sections were dried before

being stained with cresyl violet acetate (0.1% w/v) and cover-slipped with DPX mountant (VWR).

Adenoviral vector production

The cDNA clone of the rat OTR in pcD2 was generously provided by Dr. Stephen Lolait, University of Bristol (Jeng *et al*., 1996). The OTR was amplified from pcD2 using Phusion High-Fidelity DNA polymerase (New England Biolabs) and primers OTR_F (5'-GTCTCGAGCATGGAGGGCACGCCAGCA-3') and OTR_R (5'-GCCGGATCCTCATGCTGAAGATGGCTGA-3'). The PCR product was digested with XhoI and BamHI and ligated into compatible restriction sites of adenoviral vector pacAd5.CMV.IRES.GFP (Cell Biolabs). Adenoviral vector pacAd5.CMV.GFP was used as a control. The adenoviruses were generated by co-transfection of viral shuttle and backbone (pacAd5 9.2-100) vectors in HEK293T cells by calcium phosphate method in accordance with manufacturer's guidelines (Cell Biolabs). Adenoviruses were purified by two rounds of CsCl ultracentrifugation and desalted using Slide-A-Lyzer dialysis cassettes (Pierce). The purified viruses were aliquoted and stored at -80^{o}C. The virus titers were determined in triplicate by standard plaque assay.

Transfection

Ten days after fitting the telemetry device, unilateral injection of Ads into the PVN of rats was performed under combined ketamine xylazine anesthesia. The head of the rat was mounted in the stereotaxic frame and the skin was incised 3 mm to expose the scull. The stereotaxic coordinates of PVN (AP = 1.8 mm caudal from bregma, LAT = 0.4 mm from midline) were derived from the rat brain atlas (Paxinos and Watson, 2005). A glass micropipette was slowly positioned at 7.6 mm beneath the skull for infusion of virus (titer

$4 \cdot 10^{10}$ pfu/ml) in **50 nl** pressure injected in one minute. **In sham rats, a glass micropipette was slowly positioned at 7.6 mm beneath the skull.** After removal of micropipette, the skin above trepanation was sutured and sprayed with antibiotics (neomycin and bacitracin). Immediately after transfection, **a guide cannula was positioned 6.5 mm beneath the scull in n=6 rats, for microinfusion of OTR antagonist**. In the post-operative period rats were treated with gentamicin (25 mg/kg i.m.) one day before, on the day of surgery and 2 days after surgery and carprofen (5 mg/kg/day, s.c.) on the day of surgery and two days after. Rats were left to recover for seven days, time necessary for maximal expression of transfected gene (Lonergan T *et al*., 2005).

Evaluation of OTR expression

Tissue preparation & collection. Brains were snap-frozen immediately aftyer sacrifice. Hypothalamus PVN caudal-rostral tissue sections (60µm thick), were obtained using a cryostat (Leica Microsystems CM1900 Cryostat) maintained between -18 ºC and -20 ºC. Nuclei were located via the use of Toluidine blue (0.1% in 70% EtOH) staining, using a brain map (Wilson & Paxinos, 2006) for reference. Bilateral tissue punches of left and right PVN were taken by micro-punch (0.1mm diameter, Fine Science Tools) and stored in RNase-free Eppendorf tubes at -80 ºC until extraction.

RNA extraction. Tissue samples were mechanically homogenised in 1 ml QIAzol Lysis Reagent (Qiagen, Cat no. 79306) and allowed to stand for 5 min at room temperature (RT) before centrifugation (10,300 rpm, 4 ºC) for 10min. Supernatants were collected and transferred into fresh eppendorf tubes. Extraction was conducted using 200µl Chloroform (≥99%, with amylenes as stabiliser, Sigma Aldrich) and samples were centrifuged for 15 min (11,200 rpm, 4 ºC), after which the aqueous phase was removed and transferred to a fresh Eppendorf where 1 volume (350µl) of 70% (v/v) EtOH was added to precipitate total RNA.

RNA purification was performed using an RNeasy Mini Kit (Qiagen, Cat No. 74104). 700µl (total solution) was added to RNeasy Mini Kit Spin Columns for extraction via this method. The RNA was eluted from the column membrane by directly adding 30µl of RNase-free water, after which a final 1 min spin was performed (9,600rpm, RT) to collect the RNA-containing wash-through. Purified RNA was stored at -20 ºC until cDNA synthesis. RNA yield was quantified using an Implen Geneflow Nanophotometer at 390nm.

cDNA synthesis. Using 100 ng of RNA, cDNA synthesis was carried out using a QuantiTect Reverse Transcription Kit (Qiagen, Cat No. 205313). cDNA samples were then diluted to a concentration of 2ng/uLfor use in qPCR.

RT-qPCR plate loading & analysis.

ANDY - needs rewriting. Need primer sequences. Need concentrations of reagents.

What is RPL19? - define

2µl of cDNA (2ng/uL) was combined with 23µl of Mastermix (for RPL19 - 12.5µl Sybr Green, 0.1µl Forward Primer, 0.1µl Reverse Primer, 10.3µl RNase-free water; per reaction; for OXT-R 2.5ul of primer (both F&R pre-mixed) used per reaction with 12.5ul Sybr Green) were loaded into individual wells of a 96 well PCR plate (FrameStar PCR Plate, Rainin Instrument, LLC). Once loaded with all samples the plate was covered with a clear adhesive seal (Raining Instrument, LLC) and spun to ensure proper mixing of reagents. RT-qPCR analysis was then performed using an Applied Biosystems 7500 Real Time PCR System and Applied Biosystems 7500 SDS 1.2 software. Data generated were manually exported into a Microsoft Excel spreadsheet where statistical analysis was carried out. Further analysis was performed in Graphpad.

What analysis??

Pilot experiments

Pilot experiments were performed to determine the selective dose of OTR antagonist. Six rats equipped with radiotelemetric device and intrahypothalamic cannula were used. Following vehicle application (200 nL/min 0.9% NaCl), increasing doses of oxytocin (30 ng, 100ng and 300 ng) in a volume of 200 nL were microinfused for 1 minute in PVN of conscious rats, at 2 hours interval. Cardiovascular parameters were recorded between OT administration for 60 minutes. Five days later, non-peptide OTR antagonist (OTX) and OT were co-administered to rats to test OTX blocking efficacy. Subsequently, cardiovascular parameters were recorded for 60 minutes.

Experimental design

Seven days post-transfection, rats were subjected to experimentation. All experiments started around 10 a.m. in quiet surroundings under controlled environmental conditions, following 60-minute-long baseline recordings of rats housed individually in Plexiglas cages (30 cm x 30 cm x 30 cm). Cardiovascular parameters were recorded for 20 minutes under baseline conditions and 10 minutes during exposure of rats (n=6) to stress and during recovery period until normalization of BP and heart rate (HR). **Stress was induced by directing air-jet bottle compressed under 1 bar, to the top of rats' head. A separate group of wild type rats (n=6) and transfected rats (n=6) equipped with radiotelemetric device and intrahypothalamic cannula for drug injection, were subjected to microinfusion of OTX (30 ng/200 nL; n=6) or saline (200nL/min; n=6) to be recorded under baseline conditions for 20 minutes and 10 minutes during exposure to stress.**

Cardiovascular signal processing and analysis

Arterial blood pressure was digitalized at 1000 Hz in Dataquest A.R.T. 4.0 software, (DSI, St. Paul, MN, USA). Systolic BP (SBP), diastolic BP (DBP), **mean BP (MBP)** and pulse interval (PI) or its inverse, heart rate (HR), were derived from the arterial pulse pressure as maximum, minimum, integral of the arterial pulse pressure wave and inter-beat interval of the arterial pulse pressure wave, respectively. For each registration period mean value of SBP, MBP, DBP, HR and PI was calculated, and again averaged for the whole experimental group (values shown in tables and graphs).

Evaluation of the spontaneous baro-receptor reflex by the method of sequences

The method is explained in details elsewhere (Bajić *et al*., 2010). Briefly, a spontaneous baro-receptor reflex sequence is a stream of consecutively increasing/decreasing SBP samples, followed by a stream of increasing/decreasing PI interval samples delayed by 3, 4 or 5 beats in respect to SBP. A threshold for sequence length was set to four beats (Lončar-Turukalo *et al*., 2011). The sensitivity of baro-receptor reflex [BRS, ms/mmHg] was assessed as a linear regression coefficient averaged over all identified sequences (PI=BRS·SBP+const, where fitting of the curve is done in a least square sense).

Spectral analysis of BP and HR

Before spectral analysis was performed, SBP, DBP and HR signals were re-sampled at 20 Hz and subjected to nine-point Hanning window filter and linear trend removal (Milutinović *et al.,* 2006; Stojičić *et al*., 2008). Spectra were obtained using a fast Fourier transform (FFT) algorithm on 30 overlapping 2048 point time series involving in 410-s registration period of SBP, DBP and HR. The power spectrum of **BP (mmHg^2) and HR**

(bpm^2) for 30 FFT segments was calculated for the whole spectrum (total volume, TV: 0.019-3 Hz) and in three frequency ranges: very low frequency (VLF: 0.019-0.2 Hz), low frequency (LF: 0.2-0.8 Hz) and high frequency (HF: 0.8-3 Hz) range. The low frequency (LF) oscillation of SBP and DBP spectrum (LF-SBP and LF-DBP) and LF/HR-HR are markers of sympathetic activity directed to blood vessels and sympatho-vagal balance to the heart, respectively (Japundzic-Žigon, 1998).

Tissue preparation and immunohistochemistry

Anesthetized rats were trans-cardially perfused with 100 ml of 0.1 M phosphate-buffered saline (PBS pH 7.4) at room temperature followed by 300 ml of 4% (w/v) paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed, stored and cryoprotected in fixative containing 20% sucrose overnight at 4°C and subsequently frozen at -80°C. Coronal sections (35μm) of the entire rostro-caudal axis of the forebrain were sectioned on a cryostat. The free-floating sections were collected in 24-well tissue culture plates containing PBS before being processed for immunohistochemical detection of OTRs.

For immunohistochemical detection **of OTR we used commercially available goat polyclonal anti-OTR antibody (1:100, Santa-Cruz, cat. nº sc-8103)**. Free-floating rat hypothalamic sections were incubated for 30 minutes in a blocking solution comprising **10% normal horse serum for OTR (NHS; Sigma),** and 0.3% (v/v) Triton X-100 (Sigma) in 0.1 M PBS followed by rinses (3x10min) in PBS. Sections were then incubated in **goat anti-OTR primary antibody (dilution 1:100) in PBS containing 1% (v/v) NHS and 0.3% (v/v) Triton X-100 overnight**. After the primary antibody incubation, sections were rinsed in PBS (3x10min) before a 1-hr incubation in PBS containing **donkey Alexa Fluor 594 anti-goat IgG (dilution 1:100, Abcam), 1% (v/v) NGS and 0.3% (v/v) Triton X-100 at room temperature.** Following rinses in PBS (3x10min), and subsequent to further washes (3×10

min), sections were incubated for 30 minutes in **3,3'-diaminobenzidine** solution, rinsed three times in PBS and then mounted onto glass microscope slides with 0.5% (w/v) gelatin and allowed to air dry for several minutes. Once dry, the slides were dehydrated in ethanol (75%, 85%, 96% v/v), cleared in Histoclear (RA Lamb), and cover slipped in DPX mountant (VWR).

Drugs

Ketamine, xylazine, carprofen (Rimadyl®) and combination of embutramide plus mebezonium plus tetracaine (T61®) injections were purchased from Marlo Farma (Belgrade, RS). Gentamicin (Gentamicin®) injections and bacitracin plus neomycin spray (Bivacyn®) were purchased from Hemofarm (Vrsac, RS).

Statistics

Cardiovascular parameters are presented as mean ± standard error of the mean. Multiple comparisons between experimental groups were performed by ANOVA for repeated measures followed by post *hoc* Bonferroni test using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was considered at $\boldsymbol{p}<0.05$.

Results

Verification of microinjection sites and Ad expression

The position of the micropipette and guide cannula in the PVN at the end of each experiment was verified histologically (Figure 1). Ad expression was verified by analysis of eGFP **and OTR immunohistochemistry (Figure 2).**

OTR qPCR??

Pilot experiments

The dose response with OT shown in Figure 3 was performed in n=6 conscious Wt rats. OT microinfused into the PVN at a dose of 30 ng/200nL/min did not affect SBP, DBP, MBP and HR, while OT in doses of 100 ng/200nL/min and 300 ng/200nL/min induced statistically significant and comparable increases in SBP, DBP, MBP and HR over the duration of 30 minutes. The hypertensive effect and tachycardia induced by 100 ng/200nL/min OT was prevented by pre-treatment of rats by non-peptide and selective OTR antagonist (OTX) in a dose of 300 ng/200nL/min. OT infused alone in a dose of 100 ng increased SBP from 132± 2 mmHg in saline treated rats to 152 ± 1 mmHg ($p<0.01$) or in 300 ng OTX pretreated rats to 135 ± 4 mmHg ($p<0.05$). DBP changed from 80 ± 4 mmHg in saline treated rats to 93 ± 5 mmHg in 100 ng OT treated rats ($p>0.01$) or 73 ± 5 mmHg in OTX pre-treated rats ($p<0.05$) while MBP changed from 80 ± 4 mmHg in saline treated rats to 93 ± 5 mmHg in OT treated rats ($p<0.01$) to 94 ± 2 mmHg in OTX pre-treated rats ($p>0.05$). HR increased from 363 ± 14 bpm to 446 ± 18 bpm in 100 ng OT treated rats ($p<0.01$) or to 395 ± 31 bpm in OTX pretreated rats ($p>0.05$).

Cardiovascular parameters in rats over-expressing OTRs in the PVN

Under baseline conditions and during exposure to stress mean values of SBP, MBP, DBP, HR and BRS did not differ between sham injected Wt and eGFP rats (table 1). In contrast, rats over-expressing OTRs in PVN (OTR group) exhibited increased values of SBP, MBP and enhanced BRS compared to eGFP and Wt controls. Exposure of rats to air-jet stress increased SBP, MBP, DBP and HR in all groups. In Wt and e-GFP rats, a decrease in BRS occurred whilst in OTR rats **BRS did not decrease** (table 1).

Spectral analysis of cardiovascular short-term variability revealed that under basal physiological conditions BP short-term variability was comparable between Wt and e-GFP rats (Figure 4). However, in OTR rats, reduction of SBP and DBP total variability due to the statistically significant decrease of VLF variability was observed. Concomitantly, HF-SBP and HF-DBP variability increased.

When rats were exposed to air-jet stress, BP variability increased due to the increase of variability in all spectral bands. However, the increase of LF and HF variability in SBP and LF in DBP spectra was statistically significantly smaller in OTR rats as compared to both eGFP and Wt groups (Figure 4).

Under baseline physiological conditions, HR variability in rats over-expressing OTRs in PVN did not differ from e-GFP or Wt controls (Figure 5). However, when rats were exposed to acute stressful conditions, OTR rats exhibited an increase of HR variability in all spectral bands without changes in LF to HF ratio (Figure 5) suggesting a concomitant sympathetic and vagal stimulation of the heart.

Effect of OTX on cardiovascular parameters in Wild type rats and rats over-expressing OTRs in the PVN

In wild type rats, under baseline physiological conditions, microinfusion of OTX into the PVN significantly reduced BRS in respect to non-treated rats, and had no effect on mean levels of SBP, DBP, MBP and HR (table 2). In these rats, LF-SBP LF-DBP and HF-SBP spectral domains increased (Figure 6). HR variability was not affected significantly by OTX under baseline physiological conditions (Figure 7).

Wild type rats pre-treated by OTX and exposed to stress exhibited similar increase of SBP as non-treated rats, but the increase in MBP, DBP and HR were smaller and the BRS remained reduced (table 2). In these rats SBP and DBP variability

(Figure 6) and HR variability increased in all spectral domains, as well as the ratio LF/HF-HR (Figure 7).

In rats over-expressing OTR, microinfusion of OTX under baseline conditions reduced BRS in comparison to both non-treated OTR rats and Wt rats (table 2). OTX had no effect on mean values of SBP, DBP, MBP and HR of OTR rats (table 2). In these rats, both under baseline and stressful conditions, the increase in SBP, DBP (Figure 6) and HR variability (Figure 7) occurred in all spectral domains, and this was more pronounced than in non-transfected, wild type rats pre-treated with OTX. Also, in rats over-expressing OTRs in PVN and pre-treated with OTX, the increase of LF/HF-HR occurred both under baseline and stressful conditions (Figure 7).

Discussion and Conclusions

The finding of the present work suggest that OTRs in PVN tonically modulates autonomic cardiovascular control both under baseline and stressful physiological conditions. In wild type rats, application of OTX under baseline conditions reduced BPS and un-buffered BP variability. When exposed to stress, BRS remained reduced and HR variability increased in these rats, pointing to the domination of sympathetic control of the heart. In rats over-expressing OTRs, the increase in BRS was noted under baseline conditions and remaind increased during exposure to stress. These findings suggest that ectopic OTRs are functional and that the increase of their number may potentiate the physiological effects of naturally occurring ligand in PVN at physiological concentrations. Furthermore, application of OTX to OTR rats confirmed the functionality of ectopic OTRs, i.e. OTX reduced BRS and debuffered cardiovascular short-term variability under baseline and stressful conditions, and these effects were clearly more pronounced in OTR rats that in Wt rats.

It is well established that OT receptors are normally expressed in the PVN (Van Leeuwe *et al.,* 1985; Freund-Mercier *et al*., 1987; Tribollet *et al*., 1988; Yoshimura *et al.,* 1993; Adan *et al*., 1995). Electrophysiological studies showed that OT receptors in the PVN have an important function as part of an endogenous autocontrol mechanism (Richard *et al*., 1997). For instance, during suckling somato-dendritically released OT was found to stimulate OTRs on magnocelluar neurons in the PVN to increase the basal firing rate and establish a periodic bursting activity pattern. The underlying mechanisms involve priming of OT neurons and release of calcium from IP_3-sensitive intracellular stores (Inenaga and Yamashita, 1986; Moos and Richard, 1989; Richard *et al*., 1997; Ludwig and Leng, 2006). Here, we provide evidence that a change of expression of OTRs in the PVN in conscious rats can modulate neurogenic control of the circulation. Anatomical and electrophysiological studies indicate that 40% of the spinally projecting PVN neurons contain mRNA for OT **(Pyner, 2009)** and that they project to NTS, DVN, NA, RVLM and IML column of the spinal cord (Sawchenko and Swanson, 1982; Lang *et al*., 1982; Zerihun and Harris, 1983; Hosoya *et al*., 1995; Jansen *et al*., 1995; Hallbeck *et al*., 2001; Geerling *et al*., 2010) where vagal and sympathetic outflow to the heart and the blood vessels is set. Functional studies by Russ and Walker (1994) revealed that exogenously applied OT enhances the BRS, and Higa and associates (2002) also reported that microinjections of OT in the NTS facilitate reflex bradycardia via the stimulation of OT receptors. Moreover in mice lacking OT gene and OT peptide, Michelini and collaborators (2003) reported blunted BRR in response to pressure changes. **In our experiments over-expression of OTR in PVN enhanced BRS, and this effect on BRR was unmasked by OTX administration to wild type rats as well. Although our experiments cannot indicate the identity of the transmitter, there is a possibility that OTRs located on magnocellular neurons in the parvocellular part of PVN that project to cardiovascular centres in the medulla and the spinal cord could be stimulated by**

locally (somato-dendritically) relased OT to enhance (auto-control) axonal release of OTX in the vicinity of NTS, where OTRs have been shown to increase BRS. Alternatively, over-expression of ectopic OTRs could have occurred on any other neuron in PVN that is integrated in neural circuitry that alter autonomic cardiovascular control (direct ipsilateral projections to IML, RVLM and contralateral projections to both) or even on astroglial cells (Doherty *et al.,* 2011) to modulate multiple neuronal activity in PVN (Tasker *et al*., 2012). It is well established that NO, GABA and glutamate are crucial, while OTX, vasopressin, dopamine, angiotensin II selectively modulate tonic PVN signal in autonomic cardiovascular control (Pyner, 2009). All together, our results suggest that, in both transfected and wild type rats, OTRs in PVN activate downstream signaling pathways in neighboring cells leading to neurotransmitter release in brainstem targets that increase BRS. They also suggest that the number of OTRs expressed in PVN may alter the level of tonic input of PVN in neurogenic cardiovascular control without alteration in ligand release.

Our results further show that up-regulation of OTRs in PVN reduces BP variability in the VLF frequency domain under basal physiological conditions. VLF-BP oscillations contribute most to overall BP short-term variability (Japundzic-Žigon, 1998), and dominate under basal conditions. They are created by multiple mechanisms acting in concert and opposition. Oscillation at ~0.1 Hz in rat originates from spontaneous myogenic activity of blood vessels (Stauss *et al*., 2009), these can be enhanced by renin-angiotensin system activation (Ponchon and Elhgozi, 1996) and counteracted by BRR, as suggested in experiments with surgical or pharmacological opening of the baro-receptor reflex loop (Japundzic *et al*., 1990; Cerutti *et al*., 1994). Therefore, in our experiments, the reduction of VLF variability could be related to enhanced BRS**. Experiments with OTX revealed that over-expressed OTRs buffer BP and HR variability in all spectral domains under**

baseline and stressful conditions and shift autonomic control of the heart towards the vagus, while the buffering effect of OTRs in PVN of wild type rats on HRV is confined to stress. In this context it is important to mention that better understanding of central mechanisms that alter BRS, BP and HR variability that are independent prognostic markers of clinical outcome of cardiovascular diseases (Mancia *et al*., 1994; Narkiewicz and Grassi, 2008), is of interest.

Our experiments indicate that pharmacological effects of OT microinfused in PVN are hypertension and tachycardia. The failure of OTX in Wt to modulate BP and HR under baseline conditions indicates that OT has no tonic physiological influences on mean level of BP and HR. Although in OTR rats SBP and DBP are increased in respect to non-transfected rats, this does not seem to be directly associated with up-regulation of OTRs in PVN, since in OTX failed to normalize them. According to the literature, both hypotensive and hypertensive effects of exogenously applied OT were reported, depending on the route of administration. OT applied via peripheral route was found to produce short-lasting hypertension followed by longer-lasting hypotension (Peterson *et al*., 1996). Centrally applied OT was reported to decrease BP (Peterson *et al*., 1996, Peterson and Uvnäs-Moberg, 2007). It was further suggested that the central hypotensive effects of OT is mediated through axonal release in locus coeruleus where OT increases the density and the affinity of α-2 adrenoceptors known to reduce sympathetic outflow (Peterson *et al*., 2005). In OT deficient mice, BP was found to be lower than in wild type mice, suggesting a tonic influence of endogenous OT on BP (Michelini *et al*., 2003). However, OT injected in RVLM or NTS or DVN was found to increase BP (Mack *et al*., 2002; Vela *et al.,* 2010.

It is well recognized that the PVN is a major site where integration of neuroendocrine and behavioural response to stress occurs (Herman and Cullinan, 1997; Dampney and Horiuchi 2003; Benarroch, 2005). Rats exposed to air-jet stress exhibited a startle reaction

followed by freezing associated with increased BP and HR. Using microdialysis Nishioka and co-workers (1998) found that stress increases the content of OT in PVN. It was also reported that specific PVN lesions, or microinjection of antagonists intracerebroventricularly or OT antisense oligonucleotides into the PVN (Callahan *et al*. 1989; 1992) attenuated the HR response to stress. **In our experiments, microinfusion of OTX in PVN of non-transfected rats prevented stress-induced DBP increase and reduced tachycardia.** In transfected rats up-regulation of OTRs in PVN did not modulate the HR response to stress, but provoked the increase in HR variability. The increase of HR variability occurred in both sympathetically and vagally mediated frequency bands. **Pre-treatment of these rats with OTX in PVN revealed that OTRs buffer BP and HR variability and favor vagal influences to the heart (according to changes in LF/HF–HR ratio). This effect of OTRs in PVN on the heart is also seen in wild type rats only during exposure to stress (Figure 5).** The increase of vagal influences to the heart during stress was reported to be useful in protecting the heart against sympathetic over-stimulation involving cholinergic NO synthesis in ventricles (Brack *et al*., 2012). This protective effect of the vagus is lifesaving during cardiac ischemia, when sympathetic over-stimulation triggers life threatening arrhythmias and sudden death. This assumption is further supported by the work of Wsol and associates (2009) who reported failure of brain OT to attenuate cardiovascular response to stress in rats survivors of myocardial infarction.

We also observed that over-expression of OTRs in PVN buffers the stress-induced BP variability response mediated by increased sympathetic outflow to blood vessels (LF) and stimulation of respiration (HF). OTX confirmed by unmasking this effect in both OTR over-expressing and wild type rats. The buffering effect on BP variability could be mediated via magnocellular neurons expressing OT and projecting to pre-Bötzinger area (Mack *et al*., 2002; 2007). However we cannot rule out the possibility that

other neurotransmitters synthesized in PVN neurons, such as vasopressin, could have affected BP variability during stress Pyner (2009). Another possibility is that stress-induced axonal release of OT in amygdala activates a subpopulation of GABA inter-neurons that inhibit neurons in medial amygdala projecting to the brainstem autonomic nuclei (Huber *et al*., 2005; Viviani *et al*., 2011; Knobloch *et al*., 2012). This would attenuate the fear response, limit sympathetic activation and pacify respiration, as reflected in HF and LF BP short-term variability. Our findings are in line with a number of animal studies that suggest that OT activates an anti-stress response (Grippo *et al*., 2009; Lee *et al*., 2005; Windle *et al*., 1997; 2004). For instance OT is found to blunt restraint-induced hypothalamo-pituitary axes activation (Windle *et al*., 1997; 2004), to decrease cardiovascular responding to isolation (Grippo *et al*., 2009), to reduce anxiety-like behavior (Windle *et al*., 1997) and promote social interactions (Lee *et al*., 2005). In OT knock-out mice, Bernatova and co-workers (2004) described accentuated BP and corticosterone response during exposure to acute stress. In line with their finding Wsol and colleagues (2008) reported that central application of OTR antagonist enhanced BP and HR increase to environmental stress. Clinical findings in humans also support a role for OT as an anti-stress hormone. Altemus and collaborators (2001) reported that lactating women have greater parasympathetic control of the heart, and Grewen and Light (2011) found that plasma OT in lactating women is correlated with lower cardiovascular reactivity to stress.

In conclusion, our results show for the first time that OT receptors in PVN are involved in local (autocrine and/or paracrine) regulation of PVN neurons involved in tonic control of BRS and cardiovascular short-term variability. **OTRs in PVN enhance the sensitivity of the baro-receptor reflex and buffer blood pressure and heart rate short-term variability favoring vagal over sympathetic influences to the heart.** These effects are more pronounced in rats over-expressing OTR in PVN than in wild type rats. Our

findings open new perspectives in elucidating the role of OTRs in PVN in cardiovascular disease and autonomic deregulations of the circulation.

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Table 1 BP, HR and BRS in rats over-expressing OT receptors

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (bpm)	BRS (ms/mmHg)
Wt	Baseline	117 ± 5	94 ± 4	82 ± 4	340 ± 12	2.3 ± 0.2
	Stress	139 ± 6 ***	120 ± 5***	110 ± 7 ***	450 ± 21 ***	1.3 ± 0.5 *
eGFP	Baseline	118 ± 5	96 ± 4	85 ± 3	341 ± 17	2.0 ± 0.16
	Stress	138 ± 5 ***	122 ± 4***	108 ± 3 ***	448 ± 17 ***	1.3 ± 0.4 *
OTR	Baseline	134 ± 3†‡‡	106 ± 2††‡‡	86 ± 2	351 ± 14	2.9 ± 0.3 †‡
	Stress	149 ± 5 **†	115 ± 2 **‡	96 ± 3 **	430 ± 20 **	3.4 ± 0.4 ††‡‡

Values are mean of 6 rats ± s.e.m.. Wt: wild-type rats; eGFP: rats transefected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors; SBP: systolic blood pressure; SBP: systolic blood pressure; MBP: mean blood pressure; DBP: diastolic blood pressure; HR: heart rate; BRS: baro-receptor reflex sensitivity. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs.* baseline; $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$ *vs.* eGFP-transfected rats; $^{\ddagger}p<0.05$; $^{\ddagger\ddagger}p<0.01$ *vs*. wild-type rats.

Table 2 **Effects of selective OTR antagonist microinfused in PVN on BP, HR and BRS of Wilt type rats and rats over-expressing OT receptors**

		SBP (mmHg)	MBP (mmHg)	DBP (mmHg)	HR (bpm)	BRS (ms/mmHg)
Wt	Baseline	117 ± 5	94 ± 4	82 ± 4	340 ± 12	2.3 ± 0.2
	Stress	139 ± 6 ***	120 ± 5 ***	110 ± 7 ***	450 ± 21 ***	1.3 ± 0.5 *
OTX_{Wt}	Baseline	123 ± 1	98 ± 2	85 ± 2	317 ± 16	1.0 ± 0.2 ‡
	Stress	145 ± 5 ***	106 ± 3 **‡‡	87 ± 2 ‡‡	394 ± 9 ‡**	1.5 ± 0.2
OTR	Baseline	134 ± 3 ‡‡	106 ± 2 ‡‡	86 ± 2	351 ± 14	2.9 ± 0.3 ‡
	Stress	149 ± 5 **	115 ± 2 **‡	96 ± 3 **	430 ± 20 **	3.4 ± 0.4 ‡‡
OTX_{OTR}	Baseline	136 ± 5 ‡	103 ± 3 ‡	87 ± 3	323 ± 12	1.2± 0.2 ‡§§
	Stress	151 ± 6 **	111 ± 5 **‡	94 ± 1 **‡	414 ± 5 **‡	1.7 ± 0.6 §§

Values are mean of 6 rats ± s.e.m.. Wt: wild-type rats; OTX_{Wt}: Wild type rats microinfused with oxytocin receptor antagonist in PVN; OTR: rats over-expressing oxytocin receptors; OTX_{OTR}: OTR rats microinfused with oxytocin receptor antagonist in PVN; SBP: systolic blood pressure; MBP: mean blood pressure; DBP: diastolic blood pressure; HR: heart rate; BRS: baro-receptor reflex sensitivity. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs.* baseline; $^{‡}p<0.05$; $^{‡‡}p<0.01$ *vs*. wild-type rats; §§ p<0.01 *vs.* OTR rats.

FIGURE 1 **Verification of microinjection site in PVN.** Representative picture. The arrow points to the mark made by chronic cannulation. Magnification 4 x.

FIGURE 2 **eGFP fluorescence (A), immunostaining to OTRs (B) and merged (C).** Representative picture. Magnification 20 x.

FIGURE 3 **Effect of oxytocin microinfused in PVN on BP and HR**

Note that 100 ng and 300 ng of OT induced comparable increase of SBP, MBP, DBP and HR.

FIGURE 4 **Components of BP short-term variability in rats over-expressing OTRs in PVN**

In OTR rats, under baseline physiological conditions a reduction of VLF-SBP and VLF-DBP variability and increase of HF-SBP and HF-DBP variability occurred. Note smaller increase of LF-SBP, LF-DBP and HF-SBP variability in OTR rats exposed to stress in respect to controls. Values are mean ± s.e.m.. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transefected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors. Total-SBP: total systolic blood pressure variability; VLF-SBP: very low-frequency systolic blood pressure variability; LF-SBP: low-frequency systolic blood pressure variability; HF-SBP: high-frequency systolic blood pressure variability; Total-DBP: total diastolic blood pressure variability; VLF-DBP: very low-frequency diastolic blood pressure variability; LF-DBP: low-frequency diastolic blood pressure variability; HF-DBP: high-frequency diastolic blood pressure variability. **Values are mean of 6 rats ± s.e.m..** $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *vs.* baseline; $^{\dagger}p<0.05$ *vs.* eGFP; $^{\ddagger}p<0.05$ *vs*. Wt.

FIGURE 5 Components of HR short-term variability in rats over-expressing OTRs in PVN

Note that in OTR rats HR variability was enhanced in respect to control rats only during stressful conditions. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transefected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors; Total HR: total heart rate variability; VLF-HR: very low-frequency heart rate variability; LF-HR: low-frequency heart rate variability; HF-HR: high-frequency heart rate. **Values are mean of 6 rats ± s.e.m..** $p<0.05$ *vs.* baseline; $^{\dagger}p<0.05$ *vs.* eGFP rats; $^{\ddagger}p<0.05$; $^{\ddagger\ddagger}p<0.01$ *vs*. Wt.

FIGURE 6 Effects of selective OTR antagonist in PVN on the components of BP short-term variability of Wilt type rats and rats over-expressing OT receptors.

In OTR rats, under baseline physiological conditions a reduction of VLF-SBP and VLF-DBP variability and increase of HF-SBP and HF-DBP variability occurred. Also note smaller increase of LF-SBP, LF-DBP and HF-SBP variability in OTR rats exposed to stress in respect to controls. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transefected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors. Total-SBP: total systolic blood pressure variability; VLF-SBP: very low-frequency systolic blood pressure variability; LF-SBP: low-frequency systolic blood pressure variability; HF-SBP: high-frequency systolic blood pressure variability; Total-DBP: total diastolic blood pressure variability; VLF-DBP: very low-frequency diastolic blood pressure

variability; LF-DBP: low-frequency diastolic blood pressure variability; HF-DBP: high-frequency diastolic blood pressure variability. **Values are mean of 6 rats ± s.e.m..** $^{*}p<0.05$; $^{**}p<0.01$;$^{***}p<0.001$ *vs.* baseline; $^{\dagger}p<0.05$ *vs.* eGFP; $^{\ddagger}p<0.05$ *vs*. Wt.

FIGURE 7 Effects of selective OTR antagonist in PVN on the components of HR short-term variability of Wild type rats and rats over-expressing OT receptors.

Note that in OTR rats HR variability was enhanced in respect to control rats only during stressful conditions. Empty bars indicate baseline values, black bars indicate stress values. Wt: wild-type rats; eGFP: rats transefected with enhanced green fluorescent protein; OTR: rats over-expressing oxytocin receptors; Total HR: total heart rate variability; VLF-HR: very low-frequency heart rate variability; LF-HR: low-frequency heart rate variability; HF-HR: high-frequency heart rate. **Values are mean of 6 rats ± s.e.m..** $p<0.05$ *vs.* baseline; $^{\dagger}p<0.05$ *vs.* eGFP rats; $^{\ddagger}p<0.05$; $^{\ddagger\ddagger}p<0.01$ *vs*. Wt.